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Reduced Virulence of a *Listeria monocytogenes* Phospholipase-Deficient Mutant Obtained by Transposon Insertion into the Zinc Metalloprotease Gene

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A phospholipase-deficient mutant, termed JL762, was obtained from a virulent strain of *Listeria monocytogenes* by screening a bank of 5,000 Tn*1545* transposon-induced mutants on 2.5% egg yolk brain heart infusion agar. As previously shown (J. Mengaud, C. Geoffroy, and P. Cossart, Infect. Immun. 59:1043–1049, 1991), the transposon insertion took place inside the gene *mpl*, which encodes a zinc metalloprotease. By Western blot (immunoblot) analysis, we showed that loss of phospholipase activity was associated with loss of a 29-kDa zinc-dependent phosphatidylcholine-phospholipase C (PC-PLC) in culture supernatant of JL762 and of EGD-SmR incubated with ion chelator. As the parental strain, JL762 still produced in supernatants ~33-kDa proteins antigenically closely related to the 29-kDa PC-PLC. These results strongly suggest that the zinc metalloprotease of *L. monocytogenes* might play a role in the maturation of the 29-kDa PC-PLC. Although the uptake and the intracellular growth of bacteria were not affected in vitro, we found that the virulence of mutant JL762 was strongly impaired in the mouse.

Listeria monocytogenes is the prototype of the facultative intracellular bacterial pathogen capable of surviving and multiplying in mammalian cells, including macrophages (17). The sequence of events resulting in the intracellular parasitism is now clearly established. After penetrating mammalian cells, bacteria escape the phagolysosomal compartment and freely multiply in the cytosol (7, 26, 27), thus inducing actin polymerization and cell-to-cell spreading (11, 23, 30, 31). Recent genetic data have shown that listeriolysin O, a cytolytic exotoxin (9), is a major virulence factor allowing bacteria to gain access to the cytosol (2, 4, 7, 14, 21, 25). However, the virulence factors implicated in the other steps of the infectious process remain unknown. It is known that *L. monocytogenes* produces an extracellular lipolytic activity detectable around colonies grown on egg yolk agar (6, 12, 13, 24, 29, 33), which has been shown to be related to a phospholipase C (PLC) activity (15). We recently purified a zinc-dependent 29-kDa PLC (10), active mainly on phosphatidylcholine (PC) and clearly distinct from the phosphatidylinositol-PLC recently described (16, 18). The role of this 29-kDa PC-PLC as a virulence factor is unknown.

In this study, we isolated a phospholipase-deficient mutant by transposon insertion. It has been recently demonstrated that this mutant results from a single insertion of Tn*1545* inside the *mpl* gene (20), which is adjacent to *hlyA* and encodes the zinc metalloprotease of *L. monocytogenes* (5, 20). We show here that this mutant lost its capacity to produce the 29-kDa PC-PLC but still secreted cross-reactive 33-kDa proteins, suggesting that the zinc metalloprotease might be implicated in the maturation of the 29-kDa PC-PLC. We also show that virulence was strongly impaired in this mutant, although it was capable of entering and multiplying in mammalian cells.

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MATERIALS AND METHODS

Bacterial strains and culture media. Strain EGD-SmR of *L. monocytogenes*, which has been previously described (8), and *L. monocytogenes* BM4140 (serovar 7) were used as the recipient and the donor, respectively, of Tn*1545*. The phospholipase-deficient mutant isolated in this study was designated JL762 and referred to as Bug13 in a recent report (20). For subcultures, brain heart infusion (BHI) broth or agar (Biomérieux, Marcy l'Etoile, France), tryptocasein soy agar (Diagnostics Pasteur, Marnes la Coquette, France), and 5% horse BHI agar were used. For production of extracellular phospholipase, bacteria were grown in tryptic-glucose-yeast (TGY) broth prepared as follows: Biotrypticase (Biomérieux), 30 g; yeast extract (Difco Laboratories, Detroit, Mich.), 20 g; $NaHPO_4 \cdot 12H_2O$, 8.3 g; $KHPO_4$, 0.7 g; and 1,000 ml of quartz-distilled water. This broth was supplemented with 0.1 mM $ZnSO_4$ and vegetable-activated charcoal (Prolabo, Paris, France) at a final concentration of 0.2%. The charcoal-treated broth was adjusted to pH 7.5 and autoclaved at 115°C for 20 min. Sterile glucose was then added to a final concentration of 1% before inoculation.

Transposon mutagenesis and screening of phospholipase-deficient mutants. Transposon mutagenesis was achieved by transferring Tn*1545* from strain BM4140 to strain EGD-SmR as previously described (8). Insertional mutants were selected on Trypticase soy agar supplemented with streptomycin (100 μg/ml) and kanamycin (10 μg/ml). The frequency of transfer was about 10^{-8} per recipient. Multiple mating experiments were performed, and only two or three separate colonies were chosen from each plate to avoid redundance. A bank of 5,000 mutants harboring Tn*1545* was then constituted and stored at −80°C. Screening of Tn*1545* mutants was qualitatively achieved by subculturing each clone on Bacto Tryptone yeast agar (Difco Laboratories) (10 g), yeast extract (Difco Laboratories) (5 g), NaCl (5 g), agar (Difco Laboratories) (6 g), and 1,000 ml of H_2O (pH 7.3) supple-

mented with 2.5% fresh egg yolk previously diluted in 0.15 M NaCl (1:2, vol/vol). Streptomycin (100 μg/ml) and kanamycin (10 μg/ml) were added to the egg yolk medium. Colonies appeared after 1 to 3 days at 37°C and were surrounded by a large opalescent zone, revealing egg yolk degradation. The transconjugants devoid of lipolytic activity were subcultured on BHI broth supplemented with streptomycin and kanamycin, as described above. Bacterial cultures were then harvested at the end of the exponential growth phase (~10^8/ml), distributed in 1-ml lots into vials, and stored at −80°C until testing.

Phenotype analysis. Mutants were characterized by the classical tests defined elsewhere (28) and by testing fermentative capacity on 50 different substrates (API-50CH; API-System, La Balme les Grottes, France). Serotyping was performed by E. P. Espaze (Centre National des *Listeria*, Nantes, France), and lysotyping was performed by A. Audurier (Centre National de Lysotypie des *Listeria*, Tours, France). Antibiotic susceptibility was tested by the agar diffusion method on Mueller-Hinton medium (Diagnostics Pasteur). Hemolytic activity was titrated in culture supernatants of bacteria grown for 9 h at 37°C in charcoal-treated TGY, by the method of Alouf (1), with sheep erythrocytes.

Titration of phospholipase activity. Phospholipase activity was titrated in culture supernatants according to the method described by Möllby et al. (22). Briefly, 100 μl of supernatant was incubated for 1 h at 37°C with 200 μl of lecithin (3.6 g of lecithin [Merck Laboratories, Darmstadt, Germany], 2.4 g of sodium cholate, 1 mM $ZnCl_2$, 100 ml of distilled water) and 700 μl of NaCl (0.15 M). Enzymatic activity was estimated turbidimetrically at 510 nm with a Beckman M25 spectrophotometer (Brea, Calif.). One turbidimetric unit was defined as that amount of enzyme which causes an increase of absorbance of 0.100 within 1 h. Protein concentration was determined by the method of Bradford (3) with Bio-Rad reagents.

Rabbit anti-29-kDa PC-PLC serum and immunoadsorbed antibodies. Female albino rabbits (3 kg) supplied by IFFA-Credo (Saint Germain sur l'Arbresle, France), were immunized by subcutaneous injection of 25 μg of purified 29-kDa PC-PLC from *L. monocytogenes* emulsified in complete Freund's adjuvant on days 0, 7, and 14 and in incomplete Freund's adjuvant on day 90. Blood samples were collected before immunization and 2 weeks after the last injection. The preimmune serum (1:1,000) did not detect any protein in the culture supernatant of *L. monocytogenes* by Western blot (immunoblot) analysis. The antiserum was immunoadsorbed on purified 29-kDa PC-PLC as follows. The pure 29-kDa PC-PLC (75 μg/ml) was incubated on nitrocellulose sheets (BA 85; Schleicher & Schuell, Dasse, Germany) for 30 min at 22°C. After adsorption, the sheets were saturated with 5% skim milk as described above and then rinsed in phosphate-buffered saline (PBS) (pH 7.2). Rabbit anti-29-kDa PC-PLC serum diluted 1:10 (PBS, pH 7.2) was incubated for 30 min at 22°C on nitrocellulose sheets in a volume of 5 ml. After extensive washings in PBS (pH 7.2), immunoadsorbed antibodies were eluted three times with 5 ml of glycine-HCl (pH 2.2) for 15 min at 22°C. Eluates were rapidly neutralized by the addition of PO_4HNa_2 (1 M) and were concentrated on filter centricon PM 30 (Amicon) to a volume of 4 ml, corresponding to an optical density of 0.40 at 280 nm. The untreated antiserum and its immunoadsorbed derivative were then used for Western blot analysis.

Western blot analysis. Supernatants from bacterial strains grown for 9 h at 37°C in charcoal-treated (TGY) broth supplemented with 0.1 mM $ZnSO_4$ were passed through a 0.22-μm-pore-size filter and precipitated with 10% trichloroacetic acid (TCA). The precipitate was centrifuged at 10,000 × *g* at 4°C for 30 min and dissolved in 0.12 M Tris buffer (pH 6.8). Samples of 50 μl (~50 μg of protein) were boiled for 2 min in 2% (wt/vol) sodium dodecyl sulfate (SDS)–5% (vol/vol) 2-mercaptoethanol–10% (wt/vol) sucrose–0.002% (wt/vol) bromophenol blue in 0.1 M Tris hydrochloride buffer (pH 6.8). Electrophoresis (5 μg of protein per well) was performed in 13% (wt/vol) polyacrylamide-0.1% SDS in 0.37 M Tris buffer (pH 8.8) for 2 h at 150 mA. Proteins were electrophoretically transferred (1 h; 50 mA) to nitrocellulose sheets (BA 85; Schleicher & Schuell) in glycine (0.2 M)-isopropanol (20%)-0.025 M Tris buffer (pH 8.3). The sheets were incubated for 1 h at room temperature with shaking in 0.15% (vol/vol) Tween 20–PBS supplemented with 5% (wt/vol) skim milk (Regilait; France-Lait, Saint Martin-Belle Roche, France) prior to 1 h of incubation with rabbit untreated anti-29-kDa PC-PLC serum diluted 1:4,000 in the above buffer or with its immunoadsorbed derivatives (diluted 1:5-1:10) and then washed eight times in buffer before the addition of 20 ml of milk buffer containing peroxidase-labelled goat anti-rabbit immunoglobulin (Organon-Teknika-Cappel, Malvern, Pa.), diluted 1:1,000. Shaking was continued for an additional hour at room temperature, and then the filters were washed six times in buffer supplemented with 0.1% Triton X-100. Enzymatic activity was revealed by the addition of diaminobenzidine-tetrahydrochloride (Sigma) supplemented with hydrogen peroxide (0.02%).

In vitro tests of cell lines. The human colon carcinoma cell line Caco-2 was grown in Dulbecco modified Eagle medium (DMEM) supplemented with 20% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.) and 1% nonessential amino acids (Flow Laboratories, Inc., McLean, Va.) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The protocol of infection was described previously (7). Briefly, semiconfluent monolayers of Caco-2 cells were routinely inoculated with bacterial suspensions adjusted to obtain a multiplicity of infection (MOI) of 10 bacteria per cell. Penetration was allowed to proceed for 1 h at 37°C. Infected cells were then washed three times with Earle's balanced salt solution (EBSS) (GIBCO) and covered with 2 ml of Dulbecco modified Eagle medium containing gentamicin at a bactericidal concentration of 5 mg/liter in order to kill extracellular bacteria. Cells were incubated for an additional 10-h period, the starting point of which was 0 h. At various times, the cells were washed three times with EBSS and lysed with cold distilled water before viable intracellular bacteria were counted. Experiments were carried out in duplicate for each bacterial strain tested. Results were expressed as the mean $\log_{10}$ of viable bacteria per well.

The murine macrophage cell line J774 was used as follows. The culture medium was RPMI medium (GIBCO Laboratories) supplemented with 10% fetal bovine serum (GIBCO Laboratories)-1% glutamine-1% nonessential amino acids (Flow Laboratories). Cell monolayers were cultured in 25-cm^2 plastic tissue culture flasks (Corning Glass Works, Corning, N.Y.) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cell monolayers were washed once with nonsupplemented RPMI medium before infection and inoculated with bacterial suspensions adjusted to obtain an MOI of 10 bacteria per cell. Penetration was allowed to proceed for 30 min at 37°C. Infected cells were then washed three times with EBSS (GIBCO Laboratories) and covered with 2 ml of RPMI medium containing gentamicin (5 mg/liter). Cells were incubated for an additional 10-h period, the starting point of

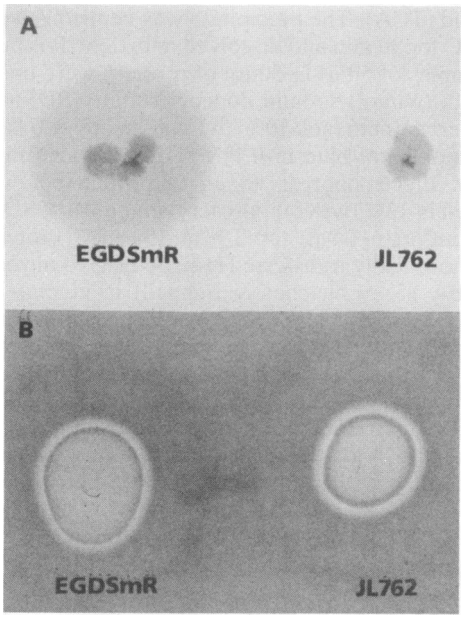

FIG. 1. Aspects of phospholipase-deficient mutant on egg yolk agar and horse blood agar. (A) Mutant JL762 and the parental strain, EGD-SmR, were grown on 2.5% egg yolk agar (for 48 h at 37°C). An opalescent halo due to the lipolytic enzyme is visible only around the parental strain. (B) Mutant JL762 and the parental strain, EGD-SmR, were grown on 5% horse blood agar. No difference in the aspect of the hemolytic zone surrounding the bacterial culture was detectable.

which was 0 h. The ensuing procedure was identical to that described above.

Virulence and histology in the mouse. Specific-pathogen-free ICR female Swiss mice (Charles River, Saint Aubin les Elboeuf, France), 6 to 8 weeks old, were used in this study. For each experiment, a vial of the frozen stock was quickly thawed, diluted appropriately in 0.15 M NaCl, and injected intravenously (i.v.) in a volume of 0.5 ml. The 50% lethal dose (LD_{50}) was determined after i.v. infection by the probit method. Bacterial growth in organs was monitored after i.v. infection by killing groups of five mice by cervical dislocation. The spleens and livers were aseptically removed and ground. Samples (0.1 ml) of serial dilutions of whole organ homogenates were surface plated on tryptic soy agar. Colonies were counted after 24 h of incubation at 37°C, and the results were expressed as the $\log_{10}$ bacterial counts per organ. Histological study was performed with 1-μm-thick sections of organs fixed in Bouin solution, embedded in paraffin, and stained with hematoxylin and eosin.

RESULTS

Isolation and phenotypic analysis of a transposon-induced phospholipase-deficient mutant of *L. monocytogenes*. By screening of a bank of 5,000 Tn*1545*-induced mutants on 2.5% egg yolk BHI agar supplemented with kanamycin and streptomycin, one stable mutant, designated JL762, was isolated. It was devoid of any lipolytic activity visible on 2.5% egg yolk BHI agar. As illustrated in Fig. 1A, an opalescent halo is visible around colonies of EGD-SmR grown for 24 to 48 h at 37°C, whereas this halo was not found around JL762 colonies grown under the same conditions. By turbidimetric assay, it was found that the enzymatic activity reached 50 U/mg of protein in the culture supernatant of strain EGD-SmR grown for 9 h at 37°C on charcoal-treated TGY broth (supplemented with 0.1 mM $ZnSO_4$). In contrast, no lipolytic activity (<1 U/ml) in the culture supernatant of mutant JL762 grown under the same conditions was detectable. Phenotypic analysis did not reveal any difference between JL762 and the parental strain, EGD-SmR, with respect to morphology, colonial aspects, motility at 22°C, catalase, D-xylose, L-rhamnose, patterns of fermentation toward 50 different carbohydrates on API-50CH, serovar 1/2a and lysotype. JL762 colonies displayed the same hemolytic phenotype on 5% horse blood agar as EGD-SmR (Fig. 1B), as well as the same hemolytic titer in supernatants of charcoal-treated broth (~2,000 U/ml). The in vitro growth curves of JL762 in BHI broth or charcoal-treated TGY broth (with 0.1 mM $ZnSO_4$) were similar to those of the parental strain (data not shown). Mutant JL762 was stable in vitro after multiple subcultures without antibiotics, and we failed to obtain revertant strains, probably because of the low frequency of Tn*1545* spontaneous loss previously estimated at 10^{-6} (8). It has been recently shown that JL762 was due to a single insertion of Tn*1545* inside the *mpl* gene encoding a zinc metalloprotease (20).

Western blot analysis of the phospholipase-deficient mutant. Bacteria from EGD-SmR or JL762 were grown for 9 h at 37°C in charcoal-treated TGY broth (with 0.1 mM $ZnSO_4$), a culture medium used to obtain optimal phospholipase activity in culture supernatants (10). TCA-precipitated proteins from supernatants were probed with a rabbit antiserum raised against a recently purified 29-kDa PLC (10) or its immunoadsorbed derivative. As shown in Fig. 2A (lane 2), the untreated antiserum diluted 1:6,000 recognized in supernatant of EGD-SmR two major bands corresponding to a 29-kDa protein and doublet 33-kDa proteins and a faint minor band at ~66 kDa. In contrast, the 29-kDa protein was absent in the supernatant of JL762, while the other bands were still detected (Fig. 2A, lane 1). This result was confirmed with the immunoadsorbed antiserum (Fig. 2B, lanes 3 and 4). The meaning of the minor 66-kDa protein detected by Western blot analysis remains unclear. It might be due either to a protein sharing common epitopes with the 29-kDa PC-PLC or to an undetectable contamination of the 29-kDa PC-PLC with a highly immunogenic protein. Moreover, when EGD-SmR was grown in charcoal-treated TGY broth (with 0.1 mM $ZnSO_4$) supplemented with 0.1 mM EDTA, the 29-kDa protein corresponding to the PC-PLC was no longer found in the culture supernatant probed with the immunoadsorbed antiserum, although the other bands persisted (Fig. 2B, lanes 1 and 2). Under these culture conditions, there is no phospholipase activity detected in supernatant of EGD-SmR (<1 U/ml), as previously described (10). These results indicate that the loss of extracellular phospholipase activity in culture supernatant of JL762 or EGD-SmR grown in EDTA was therefore associated with the loss of the 29-kDa PC-PLC.

Invasiveness and intracellular growth of the phospholipase-deficient mutant. Invasiveness and intracellular growth of JL762 were tested in vitro with Caco-2 and J774 cell lines. Cells were incubated for 30 min to 1 h at 37°C to an MOI of 10 bacteria per cell. After extensive washings, intracellular penetration and growth were studied by counting surviving bacteria associated with cells incubated in gentamicin-supplemented medium (5 mg/liter). The results are illustrated in

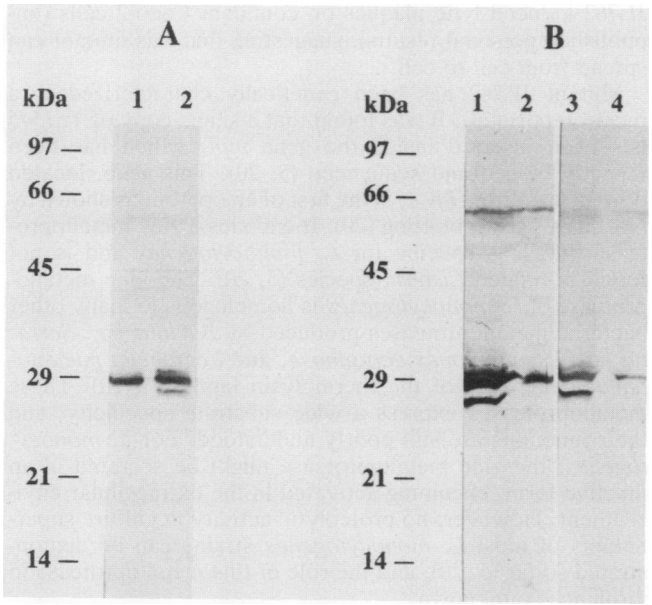

FIG. 2. Western blot analysis of culture supernatants from strain EGD-SmR and the phospholipase-deficient mutant, JL762. Bacteria were grown for 9 h at 37°C on charcoal-treated TGY broth (with 0.1 mM $ZnSO_4$), supplemented or not supplemented with 0.1 mM EDTA (as indicated below). Supernatant TCA-precipitated proteins (5 μg per well) from EGD-SmR or JL762 were studied by Western blot analysis. (A) Supernatant proteins obtained from bacteria grown without EDTA were probed with a rabbit anti-29-kDa PC-PLC (diluted 1:6,000). Lanes: 1, JL762; 2, EGD-SmR. (B) Supernatant proteins were probed with a rabbit immunoadsorbed serum anti-29-kDa PC-PLC diluted 1:5 (lanes 1 and 2) and 1:10 (lanes 3 and 4). Lanes: 1, EGD-SmR; 2, EGD-SmR plus 0.1 mM EDTA; 3, EGD-SmR; 4, JL762. It is shown that the phospholipase-deficient mutant does not produce the 29-kDa PLC but expresses the closely related cross-reactive doublet proteins of ~33 kDa. When EGD-SmR was grown in the presence of EDTA (panel B, lane 2), the 29-kDa PC-PLC is no longer detected in the supernatant, whereas the 33-kDa cross-reactive proteins persist.

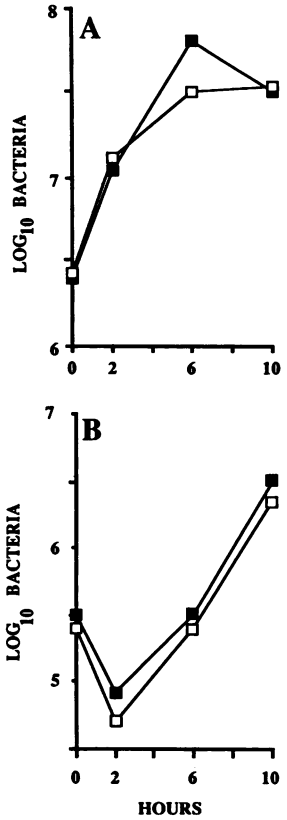

FIG. 3. Intracellular growth of the phospholipase-deficient mutant in the macrophagelike cell line J774 (A) and the enterocytelike cell line Caco-2 (B). Cells were infected at 37°C with EGD-SmR or JL762 to an MOI of 10 bacteria per cell. After extensive washings, infected cells were incubated in gentamicin-supplemented medium (5 mg/liter). Bacterial survival was monitored progressively during a 10-h period by plating samples of lysed cells onto tryptocasein soy agar. It is shown that mutant JL762 invades and multiplies in cells at the same rate as EGD-SmR. ■, EGD-SmR; □, JL762.

Fig. 3. It is shown that mutant JL762 can penetrate and multiply inside cells as well as strain EGD-SmR does.

The phospholipase-deficient mutant expresses a reduced virulence in the mouse. The virulence of JL762 was estimated by determining the LD_{50} for mice infected i.v. with increasing doses of bacteria. The LD_{50} of JL762 was found to be about $10^{7.6}$ per mouse, as opposed to $10^{6.2}$ per mouse for strain EGD-SmR. This result was confirmed by monitoring bacterial growth in organs after i.v. infection (~1×10^7 to 3×10^7 bacteria). As shown in Fig. 4, bacteria from strain EGD-SmR grew rapidly in organs to very high amounts ($\geq 10^8$ per organ), ultimately killing mice. Mutant JL762 was still capable of multiplying in host tissues at the early phase of this infection and at the same rate as EGD-SmR (Fig. 4). The rate of bacterial multiplication in organs then decreased until 48 h, and then bacteria were slowly eliminated in host tissues. Highly infective doses of JL762 (10^7 to 10^8 bacteria per mouse) induced multiple abscesses presenting the same histological aspects as those created by the parental strain (data not shown). These results indicate that the 29-kDa phospholipase-deficient mutant expresses a low level of virulence, although mutant bacteria still rapidly replicate in host tissues at the early phase of infection.

DISCUSSION

In this study, we isolated and characterized a phospholipase-deficient mutant, JL762, by transposon mutagenesis with Tn*1545*. This mutant did not release any phospholipase activity in culture supernatant and was not affected in its hemolysin production (Fig. 1), as confirmed by titration of the hemolytic activity in culture supernatant (~2,000 U/ml). The in vitro phenotypic analysis did not find any difference between JL762 and the parental strain, EGD-SmR, except for the antibiotic resistance. We then studied the capacity of JL762 to produce a recently purified and characterized 29-kDa PC-PLC (10). For this purpose, bacteria were grown in optimal conditions for the in vitro production of phospholipase activity, i.e., a 9-h incubation of charcoal-treated TGY broth supplemented with 0.1 mM $ZnSO_4$, thus allowing EGD-SmR to release phospholipase activity estimated as 50 U/ml in the supernatant. By Western blot analysis, it was revealed both with a highly diluted antiserum and with its immunoadsorbed derivative that there exist two major bands in culture supernatant of EGD-SmR, one corresponding to the 29-kDa PC-PLC and one doublet band of ~33 kDa (Fig. 2B, lane 3). In contrast, the 29-kDa PC-PLC was not detected in JL762, although the closely related cross-reactive 33-kDa proteins persisted (Fig. 2B, lane 4). Interest-

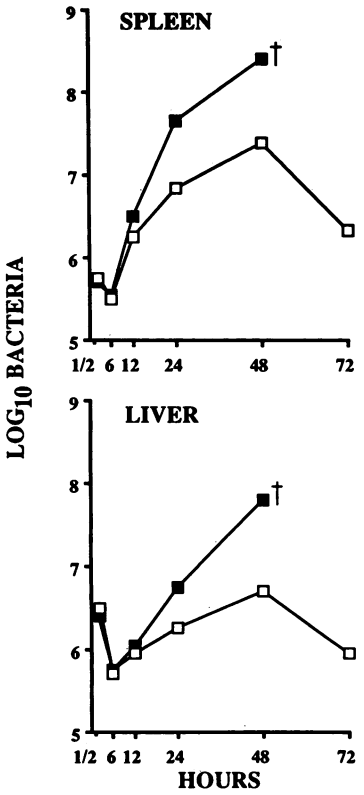

FIG. 4. Growth curves of phospholipase-deficient mutant JL762 in the spleen and liver. Groups of four to five mice were infected i.v. with lethal doses of *L. monocytogenes* parental strain EGD-SmR or JL762. Bacterial growth in the spleen and liver was then monitored. EGD-SmR (■) inoculum (i.v.), 2.8×10^7 bacteria; JL762 (□) inoculum (i.v.), 1.3×10^7 bacteria. The virulence of the phospholipase-deficient mutant was impaired, although the early rate of multiplication in host tissues was similar to that of the parental strain.

ingly, when EGD-SmR was grown in the same medium supplemented with EDTA, the culture supernatant was devoid of phospholipase activity (<1 U/ml) and the 29-kDa PC-PLC was no longer detectable by Western blot analysis, whereas the 33-kDa proteins were still present (Fig. 2B). Therefore, production of the 29-kDa PC-PLC was prevented by the addition of ion chelator. This clearly establishes that loss of phospholipase phenotype was associated with loss of the 29-kDa PC-PLC and suggests that the 33-kDa proteins might be inactive precursors of the 29-kDa PC-PLC.

The virulence of JL762 was further studied with the mouse. As shown by the LD_{50}s and by the growth curves in organs of infected mice (Fig. 4), the virulence of JL762 was significantly reduced compared with that of the parental EGD-SmR. Bacterial growth in organs was similar to that of the parental strain at the early stage of infection. The rate of multiplication was then restricted after 12 h of infection, thus resulting in a rapid elimination of bacteria from host tissues after few days (Fig. 4). With highly infective doses, JL762 could create multiple abscesses in the target organs, i.e., spleen and liver, with the same histological aspects as those seen with the parental strain. JL762 was also revealed to be capable of penetrating and rapidly multiplying in the enterocytelike cell line Caco-2 and in the macrophagelike cell line J774 as well as the parental strain does (Fig. 3). Moreover, JL762 induced lytic plaques on confluent Caco-2 cells (unpublished personal results), suggesting that this mutant can spread from cell to cell.

Mutant JL762 has been genetically characterized in a recent report (20). It was found that a single copy of Tn*1545* has been inserted inside the gene *mpl*, which has been recently cloned and sequenced (5, 20). This gene, located downstream from *hlyA*, is the first of an operon, as shown by Northern (RNA) blotting (20). It encodes a zinc metalloprotease which is specific for *L. monocytogenes* and is not found in related *Listeria* species (5, 20). The zinc metalloprotease of *L. monocytogenes* is homologous to many other bacterial metalloproteases produced by *Bacillus* sp., *Serratia* sp., *Pseudomonas aeruginosa*, and *Legionella pneumophila*, being part of the thermolysin family (5, 20). These metalloproteases express a wide substrate specificity, and their functions are still poorly understood. For *L. monocytogenes*, the zinc metalloprotease might be secreted in an inactive form, becoming activated in the extracellular environment. However, no proteolytic activity in culture supernatants of most *L. monocytogenes* strains can be demonstrated so far (5, 20), and the role of this metalloprotease in virulence is unknown.

On the basis of these recent genetic data, our results on mutant JL762 bring much new information. First, a mutation in *mpl* induces loss of the 29-kDa PC-PLC associated with a phospholipase-deficient phenotype. Second, there exist closely related cross-reactive 33-kDa proteins, enzymatically inactive, which might be precursors of the 29-kDa PC-PLC. This assumption, based on data obtained by Western blot analysis of proteins from JL762 and EGD-SmR probed with an immunoadsorbed antiserum, is further supported by recent data showing that the sequenced gene of the 29-kDa PC-PLC encodes a protein of ~33 kDa and is located in the recently described virulence operon (32). Moreover, our results demonstrate that ions are required for production of the 29-kDa PC-PLC in EGD-SmR. We know that the *L. monocytogenes* metalloprotease, as well as the 29-kDa PLC (10), is zinc dependent (5, 20). It is therefore tempting to explain our data on the basis of a zinc-dependent maturation of 33-kDa proteins by the metalloprotease. Finally, the presence of 33-kDa antigenically closely related putative precursors in JL762 strongly suggests that the transposon insertion has no major polar effect, as far as the gene of the 29-kDa PC-PLC is concerned. This might be related to the fact that a second promoter has been detected downstream from *mpl* and another upstream from *actA*, which precedes *plcB*, the gene encoding PC-PLC (19, 32). Indeed, it has been recently shown that the regulatory gene *prfA* activates the transcription of four independent genes which code for a phosphatidyl-inositol-specific PLC (gene *plcA*), listeriolysin O (gene *hlyA*), metalloprotease (gene *mpl*), and a lecithinase gene (gene *prtC*) (19, 32). Our results showing an impairment of virulence of JL762 favor the view that the 29-kDa PC-PLC might participate in the infectious process as a virulence factor. Further studies are needed to clarify the possible role of this enzyme in virulence.

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